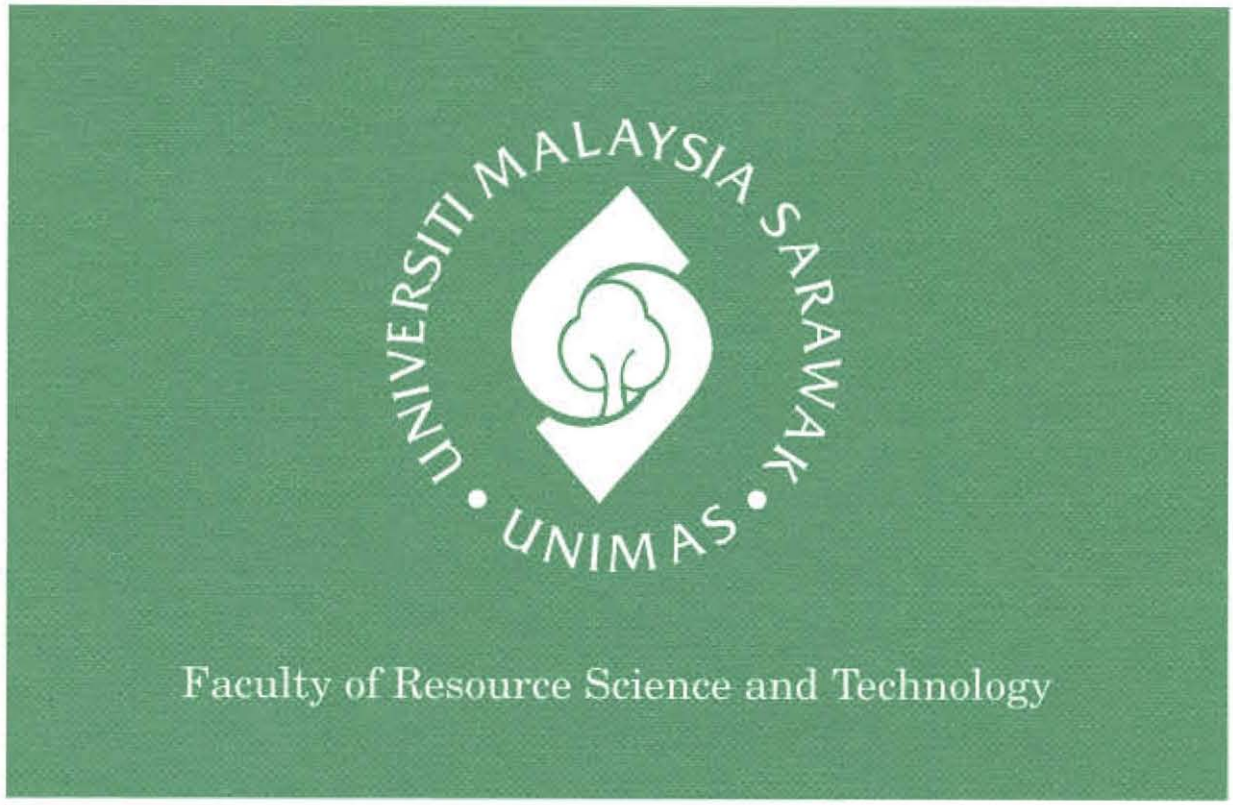

EVALUATION OF GENETIC RELATEDNESS AMONG *SHOREA PARVIFOLIA* DYER *PARVIFOLIA* ADULT TREES AND SAPLINGS USING RAPD-PCR

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LIST OF ABBREVIATIONS

CIA	Chloroform-Isoamyl Alcohol
CTAB	Centyltrimethylammonium Bromide
dH_2O	Distilled water
dNTP	Deoxyribonucleoside triphosphate
DNA	Deoxyribonucleic Acid
EDTA	Ethylene Diaminetetraacetic Acid
EtBr	Ethidium Bromide
Mg^{2+}	Magnesium ions
$MgCl_2$	Magnesium Chloride
PCI	Phenol: Chloroform: Isoamyl alcohol
PCR	Polymerase Chain Reaction
PVP	Polyvinylpyrrolidone
RAPD	Randomly Amplified Polymorphic DNA
RAPD-PCR	Randomly Amplified Polymorphic DNA- Polymerase Chain Reaction
RNA	Ribonucleic Acid
RNase	Ribonuclease
TBE	Tris-borate-EDTA
TE	Tris-EDTA buffer
T_m	Melting temperature
UPGMA	Unweighted pair-group method with arithmetic averages
UV	Ultraviolet

ABSTRACT

The genetic relatedness among *Shorea parvifolia* Dyer *parvifolia* at Semengoh Forest Reserve, Sarawak was evaluated using randomly amplified polymorphic DNA (RAPD) analysis. Four arbitrary primers that produced informative, scorable and reproducible DNA bands were selected for evaluation of genetic relatedness among the adult trees. The cluster analysis of RAPD data using unweighted pair-group method with arithmetic averages (UPGMA) grouped the 8 adult trees into 3 clusters. The result revealed that four adult trees of *S. parvifolia* Dyer *parvifolia* were not closely related and therefore selected as potential mother trees for seed production. The RAPD was used to check for misidentified in the samples as well and one sample was misidentified. For evaluation of genetic relatedness among saplings and adult trees, cluster analysis of RAPD data using UPGMA grouped the 31 samples into 3 clusters. Out of 25 saplings, only 7 saplings closely related to the selected adult trees. These results were essential in the establishment of forest Seed Production Area (SPA) and demonstrated the usefulness of RAPD marker in detecting the wrongly identified samples.

Keywords: *Shorea parvifolia* Dyer *parvifolia*, forest reserve, randomly amplified polymorphic DNA (RAPD), unweighted pair-group method with arithmetic averages (UPGMA), Seed Production Area (SPA).

ABSTRAK

Hubungan genetik antara Shorea parvifolia Dyer parvifolia di Hutan Simpanan Semengoh, Sarawak telah dinilai melalui analisis randomly amplified polymorpnic DNA (RAPD). Empat sembarang RAPD primer yang menghasilkan produk informasi, jelas dan konsisten telah dipilih untuk penilaian hubungan genetik antara pokok besar. Analisis data RAPD menggunakan unweighted pair-group method with arithmetic averages (UPGMA) telah mengelaskan 8 pokok besar kepada 3 kumpulan. Hubungan genetik antara pokok besar menunjukkan empat pokok besar S. parvifolia Dyer parvifolia adalah tidak rapat dan dipilih sebagai pokok elit untuk penghasilan bijih-benih.. RAPD analisis juga digunakan dalam pengesanan kesilapan dalam penglabelan sampel dan menunjukkan satu sampel telah salah dilabel. Bagi penilaian hubungan genetik antara pokok besar dan anak pokok, UPGMA analisis mengelaskan 31 sampel kepada 3 kumpulan. Daripada jumlah 25 anak pokok, hanya 7 anak pokok menunjukkan hubungan genetik yang rapat dengan pokok elit. Keputusan ini adalah penting untuk penubuhan kawasan penghasilan bijih-benih hutan dan menunjukkan keupayaan RAPD dalam pengesanan kesilapan dalam mengenal-pasti sampel.

Kata kunci: Shorea parvifolia Dyer parvifolia, hutan simpanan, randomly amplified polymorphic DNA (RAPD), unweighted pair-group method with arithmetic averages (UPGMA), kawasan penghasilan bijih-benih (SPA).

CHAPTER I

INTRODUCTION

Trees comprise over 90% of the biomass of the earth and serve as raw material for biofuel, fiber, solid wood products, and natural compounds as well (Han, 2001). In Malaysia, forest trees possess a great potential in the economic sector. The average tropical timber exports value by major species in Peninsular Malaysia in the year 2004 alone was approximately MYR 1160 million (Malaysia Timber Council, 2005). According to Malaysia Timber Council (2005), the export values in 2004 for sawn light red meranti were MYR 6.5 million in Peninsular Malaysia.

According to Veevers-Carter (1984), the name dipterocarp comes from the Greek meaning 'two-winged seed', dis pteron karpos. In his explanation, in South-East Asia, dipterocarps are known as keruing and meranti. He further mentioned that there are about 500 species in which 380 of them could be found in Malaysia, growing between sea-level and 800 m above it and 262 out of 380 species in Malaysia are found in Borneo.

Soerianegara and Lemmens (1994) revealed that *Shorea* is the most valuable tropical timber species in Asian. According to them, the genus consists of 194 species distributed in Sri Langka, India, Indo-China and Malaysia, in which 163 species occur in Malaysia. They mentioned that the greatest diversity are distributed in Borneo (62 Species), followed by Sumatera (23 species), Peninsular Malaysia (19 species), the Philippines (5 species) and the Moluccas (1 species). They further mentioned that it is divided into light red meranti and dark red meranti based on the specificity of the hardwood.

Shorea parvifolia Dyer is further divided into two subspecies, namely *parvifolia* and *velutinata*. According to Newman *et al.* (1996), *S. parvifolia* is locally known as meranti sarang punai or light-red meranti, meranti samak (Sarawak) and seraya punai (Sabah). *S. parvifolia* is the commonest diterocarp species in Malaysia and growing on a variety of well-drained clay soils between the sea level and 800m above it (Soerianegara & Lemmens, 1994). Therefore, it is the main source of light red meranti in South-East Asia.

The study of the genetic relatedness among individuals is a complex task but it can be accomplished by using randomly amplified polymorphic DNA (RAPD) technique developed by William *et al.* (1990), Welsh and McClelland, (1990). RAPD is a polymerase chain reaction (PCR) technique that relies on the generation of amplification products for a given nucleic acid using an amplification-based scanning technique driven by arbitrary priming oligonucleotides (Dassanayake & Samaranayake, 2003).

DNA-based markers have been widely used as an effective tool in the assessment and identification of the genetic relatedness among germplasm in many plant species, providing genetic information between species and individual and contributing to the evolutionary and ecological studies (Gepts, 1993; Weising *et al.*, 1995; Hillis *et al.*, 1996). Besides, the RAPD work with arbitrary markers without prior knowledge of the DNA sequences, simpler, convenient and rapid, safe, requires small amounts of DNA, less costly and less labour intensive than other methodologies (Caetano-Anolles *et al.*, 1991; Hadry *et al.*, 1992). Moreover, RAPD is free of environmental modulation and can be applied to analyze almost any organism even genetic information is not available (Fracaro *et al.*, 2004). This molecular marker has been successfully used in the evaluation of the genetic relatedness in teak

(Norwati *et al.*, 1995), *Tripsacum* spp. (Li *et al.*, 1999), *Origanum* spp. (Gounaris *et al.*, 2002) and *Cunila galioides* (Fracaro *et al.*, 2005).

According to Veevers-Carter (1984), dipterocarps has a very slow rate of expansion. This is due to the factors that seed dispersal and pollination of dipterocarps do not assist by wind and animals, rare flowering, no cross-breeding between different species and the threat posted by scavenger for their high oil content seeds. He added that overexploitation of timber due to its high commercial values has resulted in the massive reduction of the dipterocarps populations. Therefore, a continuous supply of genetically improved planting materials is essential to facilitate the expansion of *S. parvifolia* plantations.

This could only be done through the combination of conventional and modern breeding methods. For modern breeding method, utilization of genetic variation is an important factor for consideration of selection of mother trees for an advance breeding programme (Haines, 1994). According to Wickneswari and Ho (2003), proper and fully understanding of the genetic structure is a prerequisite for an advance breeding programme to excavate genetically improved planting materials prior to plantation establishment, for conservation purposes or management of natural resources as well.

Gene flow refers to the genetic exchange due to the migration of fertile individuals or gametes between populations (Campbell & Reece, 2002). According to Slatkin (1985), restricted gene flow would cause inbreeding depression and subsequent elimination of a population particularly under a stressful environment. In addition, restricted gene flow would possess a threat to the viability of populations of outcrossing plants (Wickneswari & Ho, 2003).

On the other hand, according to Hamrick and Nason (2000), extensive gene flow tends to reduce the variation between populations but increase genetic variation within the population. Hence, in order for selection of high quality planting materials, the genetic relatedness between planting materials should be determined prior to any subsequent breeding activities in order to enhance the genetic variation which would otherwise resulted in inbreeding depression.

The principal goal of the study is to evaluate the genetic relatedness of selected adult trees and saplings of *Shorea parvifolia* Dyer *parvifolia* at Semengoh Forest Reserve using RAPD markers.

CHAPTER II

LITERATURE REVIEW

2.1 Species review

2.1.1 Dipterocarps

According to Veevers-Carter (1984), the name 'dipterocarp' comes from the Greek meaning 'two-winged seed', dis pteron karpos. In his explanation, dipterocarps are known as keruing and meranti in South-East Asia. He described that dipterocarps are the giant trees in the lowland rain forests of South-East Asia, characterize by its significance height and is relatively easy to be observed since dipterocarps stem stretch upward and is branchless, due to its self pruning property and it branches only at the terminal of the shoots, which cause them to dominate in the upper canopy.

Veevers-Carter (1984) reported that there are about 500 species in which 380 of them could be found in Malaysia, growing between sea-level and 800 m above it. In his explanation, two hundred and sixty two out of 380 species in Malaysia are found in Borneo, but Borneo was not the origin of dipterocarps. He explained that dipterocarps are origin of Africa, drifting northwards toward India approximately 40 million years ago. He further mentioned that from India, it started to spread eastward and arrived at South-East Asia.

According to Veevers-Carter (1984), although dipterocarps are gigantic, but it is efficient to grow on poor, heavily leached soil. Thus, it implies that the trees do not depend

on the soil conditions. He explained that this is due to the symbiosis interaction between the dipterocarps and certain fungus, known as mycorrhiza, or 'fungus-root'.

Although dipterocarps means two-winged seed, it does not necessarily reflect all the members of this family are two-winged seed since some dipterocarps seed have three or five wings (Veevers-Carter, 1984). Veevers-Carter (1984) revealed that the wind plays a role in the direction of the seed dispersal, but not in the context of seed dispersal, since the wind in the equator is weak. On the other hand, he mentioned that seed dispersal by animals and insects rarely occurred since dipterocarps take years for flowering

As explained by Veevers-Carter (1984), dipterocarps take about 60 years before it initiate flowering and it will only flowering again after 3, 7 or even 11 odd-year. He further mentioned that cross-pollination or inter-breeding among different species does not occur, thus each species retains its distinct characteristics.

2.1.2 *Shorea spp.*

Soerianegara and Lemmens (1994) revealed that *Shorea* is the most valuable tropical timber species in Asian. According to them, the genus consists of 194 species distributed in Sri Langka, India, Indo-China and Malaysia, in which 163 species found in Malaysia. Borneo has the greatest diversity (62 species), followed by Sumatera (23 species), Peninsular Malaysia (19 species), the Philippines (5 species) and the Moluccas (1 species).

According to Soerianegara and Lemmens (1994), the genus is divided into two groups based on the specificity of the hardwood. Lightweight hardwood is known as light red

meranti, the species in this group include *Shorea leprosula*, *Shorea parvifolia* (Figure 2.1) and *Shorea smithiana*. Lightweight to medium-heavy hardwood is known as dark red meranti. The species in this group include *Shorea curtisii*, *Shorea macrantha*, *Shorea ovata*, *Shorea paucifolia* and *Shorea platyclados*.

(a) (b)

Figure 2.1: *Shorea parvifolia* Dyer *parvifolia*; (a) adult tree (b) saplings

As explained by Soerianegara and Lemmens (1994), *Shorea* could grow up to 70 m tall and up to 2.55 m in diameter, with bold stem for 10-42 m and extensive branches are form at the apical shoot. According to Newman *et al.* (1996), *Shorea* leaves (Figure 2.2) are arranged in such a way that alternate from one another, simple, glabrous, pinnately veined with scalariform tertiary venation and often glaucous on the adaxial surface. They explained that the stipules are usually large and persistent. *Shorea* flowers (Figure 2.3) are secund or distichous, scented, hermaphrodite, rather crowded, and inflorescences at the apical shoots or

axillary. *Shorea* flower usually contains 15 stamens, and even up to 70 stamens in every single flower and each anthers constitute of 4 pollen sacs (Soerianegara & Lemmens, 1994). Some *Shorea* flower's ovary is sustained within a stylopodium, and the style is usually longer than the ovary (Newman *et al.*, 1996).

(a) (b)

Figure 2.2: Leaf of *Shorea parvifolia* Dyer *parvifolia*; (a) adaxial surface (b) abaxial surface

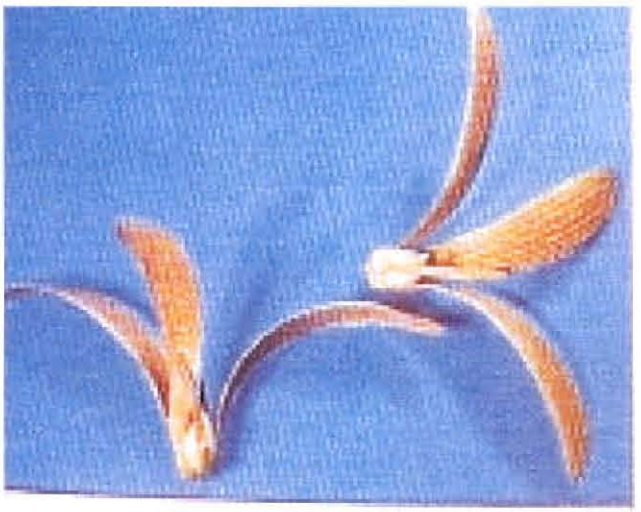

Figure 2.3: Flower seed of *Shorea parvifolia* (Adapted from Kimura and Nishiyama, 1999)

Soerianegara and Lemmens (1994) revealed that *Shorea* fruit is usually shortly stalked, with the outer 3 calyx lobes elongated and thicken and saccate at the base. They also mentioned that the nuts contained only 1 seed, subglobose to ovate in shape and free from calyx. They reported that seedling is of epigeal germination, the pericarp splitting irregularly, the young leaves are arranged spirally and often larger than the leaves of mature tree.

As explained by Newman *et al.* (1996), the bark layer is generally smooth, outer bark rather thick and brownish whereas inner bark is also rather thick but reddish, pink or orange in colour. They mentioned that Olive brown to reddish resin is liberated from the inner bark when cut and becomes opaque after exposure. They also reported that the growth ring is not present or insignificant in *Shorea*.

According to Soerianegara and Lemmens (1994), red meranti is the commonest utility for construction in Malaysia due to its non-siliceous nature. They reported that light red meranti is used for light duty flooring, fittings, paneling, ceiling, shelving, interior partitions, joinery, low-grade decking and boat planking, concrete shuttering, musical instruments, coffins, boxes, toys, turnery and matches. They further reported that several species of light red meranti produce low grade yellow dammar which is used for torches, plasters, varnishes and lacquers, solution in chloroform or xylene for preservation purposes.

2.1.3 *Shorea parvifolia* Dyer *parvifolia*

Shorea parvifolia Dyer is further divided into two subspecies, namely *parvifolia* (Figure 2.2) and *velutinata* (Figure 2.4). According to Newman *et al.* (1996), *S. parvifolia* Dyer *parvifolia* is locally known as meranti sarang punai or light-red meranti, meranti samak (Sarawak) and

seraya punai (Sabah). As explained by Soerianegara and Lemmens (1994), *S. parvifolia* is the commonest Diterocarp species in Malaysia and growing on a variety of well-drained clay soils between the sea level and 800 m above it. Therefore, it is the main source of light red meranti in South-East Asia. As explained by Newman *et al.* (1996), *S. parvifolia* could grow up to 65 m tall with branchless stem for 18-30 m and up to 1.9 m in diameter. The characteristic for the leaves are broadly ovate and thinly leathery on the adaxial surface of young leaves (Figure 2.4 (a)), large fruit calyx lobes up to 9 cm × 1.5 cm, 15 stamens in a single flower and anthers are subglobose with short appendages (Soerianegara & Lemmens, 1994).

(a) (b)

Figure 2.4: *Shorea parvifolia* Dyer *velutinata* (a) saplings (b) adaxial surface of leaf

2.2 Randomly Amplified Polymorphic DNA (RAPD)

The study of the genetic relatedness among individuals is a complex task but it can be accomplished by using randomly amplified polymorphic DNA (RAPD) technique developed by William *et al.* (1990), Welsh and McClelland (1990). RAPD is a polymerase chain reaction (PCR) technique that relies on the generation of amplification products for a given nucleic acid using an amplification-based scanning technique driven by arbitrary priming oligonucleotides (Dassanayake & Samaranayake, 2003). The unknown segments of the amplicons generated are essential, which are the uniquely characteristic of fingerprinting pattern of a specific genome. Therefore, differences in the genome can be compared and the genetic relatedness can be deduced subsequently. These differences allow any organism to be characterized at the species or the strain level (Dassanayake & Samaranayake, 2003).

DNA-based markers have provided an effective tool in the assessment and identification of the genetic relatedness among germplasm in many plant species, providing genetic information between species and individual and contributing to the evolutionary and ecological studies (Gepts, 1993; Weising *et al.,* 1995; Hillis *et al.*, 1996). Besides, the RAPD work with arbitrary markers without prior knowledge of the DNA sequences, simpler, convenient and rapid, safe, requires small amounts of DNA, less costly and less labour intensive than other methodologies (Caetano-Anolles *et al.*, 1991; Hadry *et al.*, 1992). Moreover, RAPD is free of environmental modulation and can be applied to analyze almost any organism even genetic information is not available (Fracaro *et al.*, 2004).

RAPD marker has been successfully used in the identification of commercial cultivars of broccoli and cauliflower (Hu & Quiros, 1991), evaluation of the genetic relatedness in teak

(Norwati *et al.*, 1995), *Tripsacum* spp. (Li *et al.*, 1999), *Origanum* spp. (Gounaris *et al.*, 2002) and *Cunila galioides* (Fracaro *et al.*, 2005).

2.3 Outcome of other research using RAPD technique

Despite *Shorea parvifolia* is one of the most promising plantation tree species and is seriously overexploited, the information on the genetic relationship among *S. parvifolia* trees is still scarce. There are too little emphasis on enhancing and exploring the genetic variation through biotechnological intervention. At the present, no study has been carried out on evaluating the genetic relatedness among *S. parvifolia* adult trees and its progeny using molecular markers.

The published finding is focused on evaluation of genetic relatedness among teak clones (*Tectona grandis L.*) using RAPDs marker (*Norwati et al.*, 1999). They had successfully discriminated the teak clones into three groups based on UPGMA cluster analysis and indicated high level of polymorphisms among the clones, and revealed an early indication on the good utilization of genetic variation in the teak breeding programme (Norwati *et al.*, 1999).

Study carried out by Wickneswari and Ho (2003) using simple sequence repeats (SSRs) had successfully discriminated 24 clones of *Shorea leprosula* into four distinct groups and 10 clones of *Dipterocarpus cornutus* into three distinct classes based on UPGMA cluster analysis. They had successfully distinguished four mother trees which are not closely related from each species and could be used as potential seed sources for an advance breeding programme. Besides, Wickneswari and Ho (2003) also reported that the genetic structure

among the populations were low and implied that extensive gene flow occur within each population.

Another study carried out by Fracaro *et al.* (2005) on genetic relatedness between populations of three chemotypes of *Cunila galioides* using RAPDs marker had discriminated the population into three groups sharing at a 0.5937 similarity value. The finding revealed that the populations of the citral and methene chemotypes are more closely related to each other whereas the ocimene chemotype represent a different genetic pool. The progressive substitution of the forest by herbaceous vegetation allowing genetic exchange occurred between the citral and methene chemotypes, which would explain the closer genetic relationship between the citral and methene chemotypes.

2.4 Limitations of RAPD

According to Kubelik and Szabo (1995), the absent or present of the PCR products would depend on the purity, quantity, and the quality of the DNA templates. Besides, Ellsworth *el al.* (1993) reported that the differences in the intensity of bands could also be affected by minor changes in the methodologies, such as the ratio between the DNA templates and the primer concentration, variation in the annealing temperatures, the cation concentration of PCR buffer, and magnesium ions in the reaction mixture. Moreover, Meunier and Grimont (1993) mentioned that the different lots of *Taq* DNA polymerases and the brand of the thermocycler used could also affect the RAPD fingerprinting patterns. Furthermore, the bands that demonstrated equal electrophoretic mobility might not necessarily reflect homology, and the missing band might not reflect variation because the genetic codes might be lost due to